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Plasma calprotectin and risk of cardiovascular disease: findings from the PREVEND prospective cohort study

Running Title: Plasma calprotectin and CVD risk

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Abstract

Background and aims: To assess the association of circulating calprotectin, an inflammation-associated protein, with cardiovascular disease (CVD) risk and determine whether it improves risk prediction.

Methods: Plasma calprotectin measurements were made at baseline in 5290 participants in the PREVEND prospective study. Hazard ratios (95% confidence intervals [CI]) for CVD were calculated.

Results: After a median follow-up of 8.3 years, 339 first CVD events were recorded. Calprotectin concentration was correlated with several conventional risk factors as well as with high-sensitivity C-reactive protein (hsCRP) (r=0.42). Calprotectin was log-linearly associated with CVD risk. The risk for CVD adjusted for conventional cardiovascular risk factors was 1.26 (95% CI, 1.13-1.41) per 1 standard deviation higher baseline $\log_e$ calprotectin, and was 1.24 (95% CI, 1.11-1.39) following further adjustment for triglycerides, body mass index, and other potential confounders. The association remained present after further adjustment for hsCRP 1.15 (95% CI, 1.02-1.30). Comparing extreme quartiles of plasma calprotectin levels, the corresponding adjusted HRs for CVD were 1.96 (1.37-2.82), 1.89 (1.31-2.72), and 1.56 (1.07-2.29). The association of calprotectin with CVD risk did not vary importantly in several relevant clinical subgroups. Adding calprotectin to the Framingham CVD Risk Score was associated with a C-index change (0.0016; p=0.42), difference in -2 log likelihood (p=0.038), IDI (0.0080; p<0.001), and NRI (4.03%; p=0.024).

Conclusions: There is a log-linear association of calprotectin concentration with risk of CVD, which may be partly dependent on hsCRP. Adding calprotectin to conventional risk factors improves CVD risk assessment using measures of reclassification and -2 log likelihood.

Keywords: calprotectin; cardiovascular disease; risk factor; risk prediction; cohort study

1. Introduction

Though established risk factors such as a history of diabetes, blood pressure, blood lipids, and smoking status explain a large proportion of the risk of vascular disease,[1] its pathogenesis is still not fully understood as it appears other additional factors may be involved. Accumulating evidence suggests that inflammatory processes may play an important role in the pathogenesis of coronary heart disease (CHD), which is the major manifestation of cardiovascular disease (CVD).[2,3] The development of atherosclerosis is characterised by a chronic, low-grade inflammatory process.[4] As a result, there has been an increasing interest in investigating the role of several inflammatory markers in CVD development. Several epidemiological studies have reported on the associations of both "downstream" (e.g. C-reactive protein, fibrinogen) and "upstream" (e.g. interleukins, tumor necrosis factor-α) markers with risk of CVD.[5-7] Calprotectin, also known as S100A8/A9 complex or myeloid-related protein-8/14, is an inflammatory myeloid-related protein that is mainly secreted by neutrophils.[8]

Calprotectin is considered as an acute phase protein and elevated levels have been reported in several chronic inflammatory conditions such as rheumatoid arthritis, systemic lupus erythematosus, cystic fibrosis, psoriasis, and inflammatory bowel diseases.[9-11] In addition to its role in the modulation of inflammation, leukocyte trafficking, apoptosis, and immune response; calprotectin is used as a reliable marker for the diagnosis and follow-up of inflammatory bowel diseases.[12,13] Emerging evidence suggests that calprotectin may be implicated in the pathogenesis of CVD. A number of studies have demonstrated elevated levels of calprotectin in patients with acute coronary syndromes (ACS), both at the site of coronary occlusion and in the systemic circulation, as well as in atherosclerotic plaques.[14-17] However, because the evidence from these previous studies were based on cross-sectional evaluations, the temporal nature of the relationship between circulating calprotectin and CVD is not certain. A limited number of population-based prospective studies have reported associations between increased levels of

calprotectin and increased risk of cardiovascular events. These previous reports however, were either not sufficiently powered, did not account adequately for potential confounders, or were conducted in selected populations with pre-existing CVD.[15,18,19] In addition, these previous studies did not assess the nature of the dose-response relationship between circulating calprotectin and CVD risk and did not evaluate whether the association is modified by relevant clinical characteristics. Given the uncertainties in the previous literature, we aimed to investigate in greater detail than ever before, the shape, nature, and magnitude of the prospective association between plasma calprotectin and risk of future CVD events using a population-based cohort of 5290 participants free from pre-existing CVD at baseline. We also assessed the consistency of the association in important clinical subgroups and investigated the extent to which calprotectin concentrations could improve the prediction of first-onset CVD when added to a conventional CVD risk prediction model**.**

2. Methods

2.1. Study design and population

The STROBE (STrengthening the Reporting of OBservational studies in Epidemiology) guidelines for reporting observational studies in epidemiology was used to conduct the current study (**Appendix 1**).[20] The present analyses employed the Prevention of Renal and Vascular End-stage Disease (PREVEND) study, a prospective cohort study based in a general population and which was designed to evaluate the natural course of urinary albumin excretion and its associations with renal disease and CVD. The selection of the cohort, study design, and recruitment methods have been described in previous reports.[21-25] In brief, the PREVEND cohort study is based on a representative sample of men and women living in the city of Groningen located in the Netherlands. The cohort used for this analysis comprised of 6894 individuals aged 32-80 years who were invited for the second screening phase of the PREVEND study and had their baseline assessments performed between 2001 and 2003. For the current analysis, we

used data of participants without pre-existing CVD, renal disease, or malignancy, which left a cohort of 5290 participants without missing information on plasma calprotectin, relevant confounders, and incident outcomes. The local ethics committee of the University Medical Center Groningen approved the PREVEND study protocol. Study procedures were conducted according to the Declaration of Helsinki and all participants provided written informed consent.

2.2. Assessment of calprotectin and risk markers

Study participants attended two outpatient visits during which baseline data were collected on sociodemographic characteristics, anthropometric measurements, medical history, and medication use. Additional information on medication use was collected from registries of all community pharmacies in the city of Groningen. This data source covers up-to-date information on medication use in 95% of PREVEND study participants.[26] Fasting plasma and serum venous samples were taken from participants after 15 minutes of rest prior to sample collection. Plasma samples were prepared by centrifugation at 4 ºC and sera were stored at -80 ºC until measurements were done. Plasma calprotectin levels were measured using Gentian Calprotectin turbidimetric immunoassay (Gentian, Moss, Norway) applied on a Mindray BS-400 analyser (Mindray, Shenzhen, China). HDL-C was measured by a homogeneous method (direct HDL, Aeroset System; Abbott Laboratories, Abbott Park, Illinois). Standard protocols were used to measure concentrations of total cholesterol, triglycerides, high sensitivity C-reactive protein (hsCRP), serum creatinine, and serum cystatin C and these have been described in previous reports.[27-29] Fasting plasma glucose (FPG) was measured using dry chemistry (Eastman Kodak, Rochester, New York). Estimated glomerular filtration rate (eGFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) combined creatinine-cystatin C equation.[30] Body mass index (BMI) was calculated as the ratio of the weight in kilograms to the square of height in meters.

2.3. Outcome ascertainment

The primary outcome for this analysis was first-onset composite CVD. Secondary outcomes were incident CHD and stroke events. We included all outcome events that occurred from study entry (2001-2003) to 1-1-2011. The source of data on hospitalization for incident CVD events was obtained from PRISMANT, which is the Dutch National Registry of hospital discharge diagnoses.[31] Cardiovascular deaths and their dates were ascertained by data linkage with the Dutch Central Bureau of Statistics. Outcome data were coded according to the *International Classification of Diseases*, Ninth Revision (ICD-9) until 01 January 2009 and after this date, ICD-10 codes were used. First-onset composite CVD was defined as the combined outcomes of acute and subacute ischemic heart disease (IHD), acute myocardial infarction (AMI), coronary artery bypass grafting (CABG) or percutaneous transluminal coronary angioplasty (PTCA), occlusion or stenosis of the precerebral or cerebral arteries, subarachnoid hemorrhage, intracerebral hemorrhage, other intracranial hemorrhage, and other vascular interventions such as percutaneous transluminal angioplasty or bypass grafting of peripheral vessels and aorta. Coronary heart disease was defined as fatal or nonfatal IHD, fatal or nonfatal MI, CABG, and PTCA. Stroke events were defined as occlusion and stenosis of precerebral or cerebral arteries, and carotid obstruction, subarachnoid hemorrhage, intracerebral hemorrhage, other and unspecified intracranial hemorrhage.

2.4. Statistical analyses

Skewed variables which included calprotectin were natural log transformed to achieve approximately normal distributions. Baseline characteristics were presented as means (standard deviation, SD) or median (interquartile range, IQR) for continuous variables and percentages for categorical variables. Age- and sex-adjusted partial correlation coefficients were estimated to

assess the cross-sectional correlations of plasma calprotectin levels with CVD risk markers. Time-to-event Cox proportional hazard regression models were used to compute hazard ratios (HRs) for the associations of plasma calprotectin with risk of cardiovascular outcomes, after confirmation of no major departure from the proportionality of hazards assumptions.[32] Cumulative Kaplan-Meier curves for CVD during follow-up were plotted according to quartiles of calprotectin. The shape of the relationship between plasma calprotectin and CVD risk was characterized by plotting HRs estimated within quartiles of baseline calprotectin relative to the bottom quartile versus the mean calprotectin value in each quartile using floating absolute risks (FARs).[33] Given the approximately linear shape of the calprotectin-CVD association, HRs were computed per 1 SD higher $\log_e$ calprotectin concentration. The SD of baseline $\log_e$ calprotectin level was 0.54 (equivalent to approximately 2-fold higher circulating calprotectin level, as $e^{0.54}$=1.72). We also modelled HRs as quartiles defined according to the baseline distribution of plasma calprotectin levels. The following four models were used for adjustment: (i) age and sex; (ii) other established CVD risk factors (history of diabetes mellitus, smoking status, SBP, total cholesterol, and HDL-C); (iii) other potential confounders (triglycerides, BMI, alcohol consumption, FPG, and eGFR); and (iv) hsCRP. We used interaction tests to assess statistical evidence of effect modification by several clinical characteristics. To minimize the possibility of bias due to reverse causation, we performed sensitivity analyses that excluded the first two years of follow-up, participants with a history of diabetes mellitus at baseline, or participants on lipid-lowering medication.

Finally, we assessed whether adding information on plasma calprotectin to conventional cardiovascular risk factors[34] is associated with an improvement in CVD risk prediction. To achieve this, we calculated measures of discrimination for censored time-to-event data (Harrell's C-index [35]) and reclassification. To investigate the change in C-index on addition of calprotectin to conventional cardiovascular risk factors, two CVD risk prediction models were fitted; one

based on conventional risk factors included in the Framingham CVD Risk Score (FRS) (i.e., age, sex, smoking status, SBP, total cholesterol, and HDL-C)[36] and the second model with the FRS risk factors plus calprotectin. We then evaluated whether calprotectin helps to correctly classify participants into predicted CVD risk categories. Using the American College of Cardiology (ACC) and American Heart Association (AHA) 2013 cardiovascular risk categories of low (<5%), intermediate (5 to <7.5%), and high (≥7.5%) risk,[37] reclassification was assessed using the categorical net-reclassification-improvement (NRI).[38] Reclassification analysis was based on the nine years of follow-time for this study. Finally, we calculated the integrated discrimination improvement (IDI), which integrates the NRI over all possible cutoffs of predicted risk and mathematically corresponds to the difference in discrimination slopes of the 2 models in comparison.[38] Our risk prediction analysis was restricted to study participants without known histories of CVD or diabetes mellitus at baseline. Given that Harrell's C-index is based on ranks rather than on continuous data, it can be insensitive in detecting differences.[39,40] To avoid discarding potential biomarkers that can be used in risk prediction, sensitive risk discrimination methods such as the -2 log likelihood test have been recommended.[39,40] Therefore, in addition to Harrel's C-index, we tested for differences in the -2 log likelihood of prediction models with and without inclusion of calprotectin. We conducted all statistical analyses using Stata version 14 (Stata Corp, College Station, Texas).

3. Results

3.1. Baseline characteristics

The mean age at study entry of participants was 53 (SD 12) years and 47.8% were men (**Table 1**). There were significant differences in baseline characteristics (including calprotectin levels) between participants who did and did not develop CVD. The overall median (IQR) plasma concentration of calprotectin was 0.50 (0.35-0.70) mg/L. Calprotectin concentration was weakly correlated with several risk markers: positively with age, BMI, blood pressure, total cholesterol,

triglycerides, and FPG; and inversely with HDL-C. The strongest correlation was observed with hsCRP (r=0.42). Baseline calprotectin concentrations were higher by 9% in males compared with females and by 22% in current smokers compared with never and former smokers (**Table 2**).

3.2. Plasma calprotectin and risk of incident CVD

During a median follow-up of 8.3 (IQR, 7.7-8.9) years, 339 incident CVD events (annual rate 8.22/1,000 person-years at risk; 95% CI: 7.39 to 9.14) occurred. Cumulative hazard curves showed an increased risk of CVD in the top quartile of calprotectin levels compared with the bottom three quartiles (*p*-value for log-rank test < 0.001 for all; **Figure 1**). In analyses adjusted for age and sex and also for conventional risk factors, there was a log-linear association of calprotectin with risk of CVD (**Figure 2**). **Table 3** reports the associations of plasma calprotectin with the risk of CVD outcomes. The age- and sex-adjusted HR (95% CI) of CVD per 1 SD increase in baseline $\log_e$ calprotectin was 1.38 (1.25 to 1.54) and it was 1.26 (1.13 to 1.41) following further adjustment for history of diabetes, smoking status, SBP, total cholesterol, and HDL-C. The association remained consistent on additional adjustment for triglycerides, BMI, alcohol consumption, FPG, and eGFR, but was somewhat attenuated by further adjustment for $\log_e$ hsCRP 1.15 (1.02 to 1.30). In analyses that compared the highest with the lowest quartiles of the distribution of calprotectin concentrations, the corresponding adjusted HRs for the respective models were 2.60 (1.82 to 3.71), 1.96 (1.37 to 2.82), and 1.56 (1.07 to 2.29). The findings were broadly similar in sub-analyses of CHD; however, the association was less robust for stroke and in subsidiary analyses using ischemic stroke as a specific endpoint (**Table 3**). In sensitivity analyses that excluded the first two years of follow-up, people with diabetes mellitus at baseline, or people on lipid-lowering medication, the findings were qualitatively similar (**Appendix 2**). The HRs for CVD did not vary importantly by age, sex, alcohol consumption, smoking status, BMI, blood pressure, cholesterol, eGFR, or hsCRP; except for evidence of

effect modification by FPG (*P*=0.032) (**Appendix 3**). The HR was more extreme in participants with high levels of FPG (≥ 5.0 mmol/l) compared to those with lower levels (< 5.0 mmol/l). To put the strength of the association of calprotectin levels with CVD risk into context, direct comparisons were made to associations of several established and emerging CVD risk markers. The association of circulating calprotectin with CVD risk was of comparable strength to SBP, total cholesterol, and hsCRP (**Appendix 4**).

3.3. Plasma calprotectin and CVD risk prediction

A CVD risk prediction model (FRS) containing established cardiovascular risk factors yielded a C-index of 0.8105 (95% CI: 0.7906 to 0.8305). After addition of information on calprotectin levels, the C-index was 0.8121 (0.7920 to 0.8322), representing a non-significant increase of 0.0016 (-0.0023 to 0.0055; *p*=0.42). However, when investigating differences in the -2 log likelihood of the FRS, the -2 log likelihood was significantly improved on addition of calprotectin to the FRS (*p* for comparison=0.038). Of the 1071 participants who remained free of CVD, 49 (4.58%) were correctly reclassified to a lower risk category and 28 (2.61%) were reclassified to a higher risk category. Of the 338 participants who developed CVD, 18 (5.33%) were correctly reclassified to a higher risk category and 11 (3.25%) were reclassified to a lower risk category. After taking into account inappropriate reclassification, there was a significant improvement in the classification of participants into predicted CVD risk categories (NRI: 4.03%, 0.52 to 7.54%; *p*=0.024). The IDI was 0.0080 (95% CI, 0.0044 to 0.0116; *p*<0.001).

4. Discussion

4.1. Main findings

In this large-scale population-based study of individuals without a history of CVD at study entry, baseline calprotectin levels were weakly and positively correlated with indices of adiposity, as well as lipid, metabolic, and renal function markers. A moderately strong correlation was

observed with hsCRP. The current analysis of 5290 individuals has demonstrated a log-linear association of calprotectin concentration with the risk of CVD. The initial association changed very little after adjustment for established and other emerging risk factors, suggesting that calprotectin levels are independent of such factors. However, the association was somewhat attenuated when further adjusted for inflammation as measured by hsCRP. We showed that HRs for CVD with calprotectin levels were similar in a range of clinically relevant subgroups, such as in men and women, or at different levels of established risk factors, except for evidence of effect modification by FPG. The associations were also similar in several sensitivity analyses. In investigation of other CVD endpoints, although the findings were broadly similar for CHD, that for composite and ischemic stroke events were less distinct or robust and which could be attributed to the low event rates. In direct comparisons with several established and emerging cardiovascular markers, we showed that the HR for CVD with calprotectin levels may be comparable to some of these markers. Finally, adding calprotectin to the FRS did not improve discrimination of CVD risk using Harrell's C-index; but there was a significant improvement on using the -2 log likelihood method. There was also significant improvement in reclassification of participants across clinical risk categories

4.2. Comparison with previous work

In a prospective, nested case-control validation study consisting of 255 case-control pairs among healthy postmenopausal women followed for a median time of 2.9 years, Healy and colleagues demonstrated an increased risk of cardiovascular events (nonfatal myocardial infarction or stroke, or cardiovascular death) with increased levels of calprotectin and this was independent of conventional risk factors as well as CRP.[15] In another nested case-control study of patients with ACS, the risk of cardiovascular death or myocardial infarction after 30 days increased with increased levels of calprotectin.[18] In a more recent cohort consisting of 664 middle-aged individuals, Cotoi reported independent associations of calprotectin with coronary

events and CVD death; however, the associations were more robust in women.[19] Drawbacks of some of these previous studies included (i) small sample sizes; (ii) short-term follow-up periods; (iii) inclusion of participants with pre-existing CVD; (iv) the inability to account for a comprehensive panel of other potential confounders; (v) no formal assessment of the shape of the relationship between plasma calprotectin and CVD risk was undertaken; hence it is not known if there is a dose-response relationship to the association; (vi) no subgroup analysis was undertaken to assess if the association is modified by relevant clinical characteristics; or (vii) no formal risk prediction analyses was performed. Though the general findings concur with these previous studies; to the best of our knowledge, a comprehensive assessment of the independence, magnitude, shape, and consistency of the prospective association between plasma calprotectin and risk of CVD in the general population has not been previously reported. Our findings show that the association between plasma calprotectin and increased CVD risk is consistent with a log-linear shape. Furthermore, the association is independent of several conventional risk factors as well as alcohol consumption and renal function, but was considerably attenuated but not abrogated by hsCRP. The association was also similar in important subgroups, such as men and women; which are contrary to findings suggesting that the relationship may only be important in women.[19] The null findings we observed for stroke is also consistent with that of a previous study.[19] Finally, the current study reported estimates of the metrics for risk discrimination and reclassification for the first time.

4.3. Possible explanations for findings

Findings from a number of studies have implicated calprotectin in the pathogenesis of CHD.[14,15,17] Calprotectin is an inflammation-associated protein and given that inflammation plays a major role in the pathogenesis of atherosclerosis[41] and with coronary atherosclerosis being a precursor for most coronary events; calprotectin may be involved in the pathogenesis of CHD via inflammatory processes. Our results show that the association of calprotectin with

CVD/CHD was considerably attenuated on adjusting for hsCRP (an inflammatory biomarker, which is also a strong independent predictor of CHD/CVD[5]); findings which substantiate the importance of hsCRP as a confounding factor and lend further support to the inflammation hypothesis of CVD/CHD development. Calprotectin is highly expressed or the most abundant cytosolic protein in neutrophils,[8] which are involved in the pathophysiology of coronary syndromes.[42,43] Since neutrophils are strongest independent determinants of calprotectin,[19] it has been suggested that calprotectin may be a marker of neutrophil involvement in CVD pathogenesis.[19] Evidence from clinical studies in humans and animal models suggest that neutrophils may play important roles in atherogenesis and as mediators of plaque destabilization[44-46] via endothelial activation, oxidative stress as a result of generation of reactive oxygen species, low-density lipoprotein oxidation, and apoptosis.[45-47] Morrow and colleagues also suggest that calprotectin may not only be a marker of neutrophil activation, but also be directly involved in CVD/CHD pathogenesis via inflammatory and thrombotic responses.[18] Indeed, data from animal models suggest calprotectin (i) is essential for neutrophil recruitment at sites of injury[48] and (ii) binds to receptor for advanced glycation end-products (RAGE), which trigger inflammatory and thrombotic responses[49] or stimulate the production of neutrophils and inflammatory monocytes, leading to impaired regression of atherosclerotic plaques.[50] The absence of calprotectin in hyperlipidemic mice has also been shown to be associated with delayed atherosclerosis.[51] In human studies, calprotectin has been shown to activate the vascular endothelium, impair endothelial integrity,[52,53] and is also strongly correlated with pro-inflammatory cytokines (such as interferons, interleukins, tumor necrosis factor-α),[19] which are proatherogenic and play important roles in CVD development.[44] The mechanistic evidence is still early and further studies are required to investigate any mediating effects of calprotectin on atherosclerosis and CVD development.

4.4 Implications of findings

The current findings of a log-linear, independent, and specific association of calprotectin concentration with CVD, may suggest the existence of a causal relationship; and the added prognostic value of calprotectin on top of established risk factors may have several implications for the development of CVD prevention strategies. However, further studies are needed to replicate these findings, and which will stimulate further study to help determine if calprotectin is causally involved in CVD/CHD development and whether information on calprotectin might aid in the early identification of people at high risk of future CVD. The findings also suggest that calprotectin might be involved in inflammatory processes that underlie CVD development and could be a potential new therapeutic target that can be exploited to complement lipid-lowering strategies. Indeed, findings from a previous study suggest that intensive statin therapy lowers levels of calprotectin by day 30.[18] Drugs that block the binding of calprotectin to its receptors have already been developed and approved for human clinical testing,[54,55] opening up new opportunities for CVD prevention.

4.5. Strengths and limitations

Strengths and potential limitations of the current study merit careful consideration. These new data involve many more participants and incident CVD cases than the previous studies conducted in general population settings.[15,19] Participants were identified from population registers; follow-up was long; there was minimization of potential biases by exclusion of individuals with prevalent CVD; concomitant measurements of several established and emerging risk factors enabled adjustment for a range of possible confounding factors; and the analysis was comprehensive which included assessment of the dose-response relationship, evaluation of effect modification, conducting formal risk prediction analyses using sensitive measures such as the -2 log likelihood, and testing the robustness of the findings using several sensitivity analyses. Limitations of the current study included (i) absence of data on neutrophil counts which precluded the ability to assess if the calprotectin-CVD association is independent

of or modified by neutrophil count; (ii) the inability to correct for regression dilution because of absence of repeat measurements, which could have underestimated the associations demonstrated; (iii) though the study had complete measurements of risk factors used in standard risk prediction algorithms and prevalent CVD or diabetes were excluded at baseline, participants were not adequately suitable for risk prediction and reclassification analyses as no participant was followed up for 10 years; (iv) the risk reclassification metrics (e.g., NRI) used have inherent limitations, such as dependence on the choice of cutoff and time frame for predicted risks and on the age distribution of the study population; (v) the potential for residual confounding due to other unmeasured covariates; and (vi) inability to generalize the findings to different ethnicities.

5. Conclusion

In a predominantly Caucasian population, there is a log-linear association of calprotectin concentration with risk of CVD, which is independent of established risk factors but may be partly dependent on inflammation as measured by hsCRP. Furthermore, adding calprotectin to conventional risk factors improves CVD risk assessment using measures of reclassification and -2 log likelihood. Further assessment of the role of calprotectin in CVD prevention is warranted.

Conflicts of Interest

Tom Nilsen, Clara Hidden, and Erling Sundrehagen are employees of the manufacturer of the calprotectin assay, Gentian, in Moss, Norway.

Author Contributions

JLF-G, LMK, TN, CH, ES, RPFD, and RPFD conceived and designed the study. LMK, TN, CH, ES, RPFD, and SJLB acquired data. SKK analyzed data. SKK, SS, SJLB interpreted the data. SKK drafted the manuscript. SKK, JLF-G, LMK, TN, CH, ES, SS, RPFD, and SJLB critically revised the manuscript for important intellectual content. SJLB supervised the study. SKK is the guarantor of this work, and as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis

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References

1. Wood D. Established and emerging cardiovascular risk factors. *Am Heart J.* 2001;141(2 Suppl):S49-57.

2. Libby P, Ridker PM, Hansson GK. Progress and challenges in translating the biology of atherosclerosis. *Nature.* 2011;473(7347):317-325.

3. Hansson GK. Inflammation, atherosclerosis, and coronary artery disease. *N Engl J Med.* 2005;352(16):1685-1695.

4. Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol.* 2011;12(3):204-212.

5. Emerging Risk Factors C, Kaptoge S, Di Angelantonio E, et al. C-reactive protein concentration and risk of coronary heart disease, stroke, and mortality: an individual participant meta-analysis. *Lancet.* 2010;375(9709):132-140.

6. Danesh J, Lewington S, Thompson SG, et al. Plasma fibrinogen level and the risk of major cardiovascular diseases and nonvascular mortality: an individual participant meta-analysis. *Jama.* 2005;294(14):1799-1809.

7. Kaptoge S, Seshasai SR, Gao P, et al. Inflammatory cytokines and risk of coronary heart disease: new prospective study and updated meta-analysis. *Eur Heart J.* 2014;35(9):578-589.

8. Hessian PA, Edgeworth J, Hogg N. MRP-8 and MRP-14, two abundant Ca(2+)-binding proteins of neutrophils and monocytes. *J Leukoc Biol.* 1993;53(2):197-204.

9. Bjarnason I, Sherwood R. Fecal calprotectin: a significant step in the noninvasive assessment of intestinal inflammation. *J Pediatr Gastroenterol Nutr.* 2001;33(1):11-13.

10. de Seny D, Fillet M, Ribbens C, et al. Monomeric calgranulins measured by SELDI-TOF mass spectrometry and calprotectin measured by ELISA as biomarkers in arthritis. *Clin Chem.* 2008;54(6):1066-1075.

11. Ho GT, Lee HM, Brydon G, et al. Fecal calprotectin predicts the clinical course of acute severe ulcerative colitis. *Am J Gastroenterol.* 2009;104(3):673-678.

12. Henderson P, Anderson NH, Wilson DC. The diagnostic accuracy of fecal calprotectin during the investigation of suspected pediatric inflammatory bowel disease: a systematic review and meta-analysis. *Am J Gastroenterol.* 2014;109(5):637-645.

13. van Rheenen PF, Van de Vijver E, Fidler V. Faecal calprotectin for screening of patients with suspected inflammatory bowel disease: diagnostic meta-analysis. *BMJ.* 2010;341:c3369.

14. Altwegg LA, Neidhart M, Hersberger M, et al. Myeloid-related protein 8/14 complex is released by monocytes and granulocytes at the site of coronary occlusion: a novel,

early, and sensitive marker of acute coronary syndromes. *Eur Heart J.* 2007;28(8):941-948.

15. Healy AM, Pickard MD, Pradhan AD, et al. Platelet expression profiling and clinical validation of myeloid-related protein-14 as a novel determinant of cardiovascular events. *Circulation.* 2006;113(19):2278-2284.

16. Katashima T, Naruko T, Terasaki F, et al. Enhanced expression of the S100A8/A9 complex in acute myocardial infarction patients. *Circulation journal : official journal of the Japanese Circulation Society.* 2010;74(4):741-748.

17. Ionita MG, Vink A, Dijke IE, et al. High levels of myeloid-related protein 14 in human atherosclerotic plaques correlate with the characteristics of rupture-prone lesions. *Arterioscler Thromb Vasc Biol.* 2009;29(8):1220-1227.

18. Morrow DA, Wang Y, Croce K, et al. Myeloid-related protein 8/14 and the risk of cardiovascular death or myocardial infarction after an acute coronary syndrome in the Pravastatin or Atorvastatin Evaluation and Infection Therapy: Thrombolysis in Myocardial Infarction (PROVE IT-TIMI 22) trial. *Am Heart J.* 2008;155(1):49-55.

19. Cotoi OS, Duner P, Ko N, et al. Plasma S100A8/A9 correlates with blood neutrophil counts, traditional risk factors, and cardiovascular disease in middle-aged healthy individuals. *Arterioscler Thromb Vasc Biol.* 2014;34(1):202-210.

20. von Elm E, Altman DG, Egger M, Pocock SJ, Gotzsche PC, Vandenbroucke JP. The Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement: guidelines for reporting observational studies. *Journal of clinical epidemiology.* 2008;61(4):344-349.

21. Kunutsor SK, Bakker SJ, Kootstra-Ros JE, Gansevoort RT, Dullaart RP. Circulating gamma glutamyltransferase and prediction of cardiovascular disease. *Atherosclerosis.* 2014;238(2):356-364.

22. Kunutsor SK, Kieneker LM, Bakker SJL, James RW, Dullaart RPF. Incident type 2 diabetes is associated with HDL, but not with its anti-oxidant constituent - paraoxonase-1: The prospective cohort PREVEND study. *Metabolism - Clinical and Experimental.* 2017.

23. Kunutsor SK, Kieneker LM, Burgess S, Bakker SJL, Dullaart RPF. Circulating Total Bilirubin and Future Risk of Hypertension in the General Population: The Prevention of Renal and Vascular End-Stage Disease (PREVEND) Prospective Study and a Mendelian Randomization Approach. *Journal of the American Heart Association.* 2017;6(11).

24. Kunutsor SK, Bakker SJ, Gansevoort RT, Chowdhury R, Dullaart RP. Circulating total bilirubin and risk of incident cardiovascular disease in the general population. *Arterioscler Thromb Vasc Biol.* 2015;35(3):716-724.

25. Kunutsor SK, Bakker SJ, James RW, Dullaart RP. Serum paraoxonase-1 activity and risk of incident cardiovascular disease: The PREVEND study and meta-analysis of prospective population studies. *Atherosclerosis.* 2015;245:143-154.

26. Visser ST, Schuiling-Veninga CC, Bos JH, de Jong-van den Berg LT, Postma MJ. The population-based prescription database IADB.nl: its development, usefulness in outcomes research and challenges. *Expert Rev Pharmacoecon Outcomes Res.* 2013;13(3):285-292.

27. Dullaart RP, Perton F, van der Klauw MM, Hillege HL, Sluiter WJ, Group PS. High plasma lecithin:cholesterol acyltransferase activity does not predict low incidence of cardiovascular events: possible attenuation of cardioprotection associated with high HDL cholesterol. *Atherosclerosis.* 2010;208(2):537-542.

28. Kunutsor SK, Bakker SJ, Kootstra-Ros JE, Blokzijl H, Gansevoort RT, Dullaart RP. Inverse linear associations between liver aminotransferases and incident cardiovascular disease risk: The PREVEND study. *Atherosclerosis.* 2015;243(1):138-147.

29. Kunutsor SK, Bakker SJ, Kootstra-Ros JE, Gansevoort RT, Gregson J, Dullaart RP. Serum Alkaline Phosphatase and Risk of Incident Cardiovascular Disease: Interrelationship with High Sensitivity C-Reactive Protein. *PloS one.* 2015;10(7):e0132822.

30. Inker LA, Schmid CH, Tighiouart H, et al. Estimating glomerular filtration rate from serum creatinine and cystatin C. *N Engl J Med.* 2012;367(1):20-29.

31. Stricker BH, Herings RM. [Plea for the retention of the Dutch National Medical Registration (LMR) to provide reliable information regarding public health and healthcare]. *Nederlands tijdschrift voor geneeskunde.* 2006;150(35):1916-1917.

32. Therneau TM, Grambsch PM. *Modeling Survival Data: Extending the Cox Model.* New York, NY: Springer; 2000, pp. 39-77.

33. Easton DF, Peto J, Babiker AG. Floating absolute risk: an alternative to relative risk in survival and case-control analysis avoiding an arbitrary reference group. *Stat Med.* 1991;10(7):1025-1035.

34. Greenland P, Alpert JS, Beller GA, et al. 2010 ACCF/AHA guideline for assessment of cardiovascular risk in asymptomatic adults: a report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines. *Circulation.* 2010;122(25):e584-636.

35. Harrell FE, Jr., Lee KL, Mark DB. Multivariable prognostic models: issues in developing models, evaluating assumptions and adequacy, and measuring and reducing errors. *Stat Med.* 1996;15(4):361-387.

36. D'Agostino RB, Sr., Vasan RS, Pencina MJ, et al. General cardiovascular risk profile for use in primary care: the Framingham Heart Study. *Circulation.* 2008;117(6):743-753.

37. Goff DC, Jr., Lloyd-Jones DM, Bennett G, et al. 2013 ACC/AHA Guideline on the Assessment of Cardiovascular Risk: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines. *Circulation.* 2013.

38. Pencina MJ, D'Agostino RB, Sr., D'Agostino RB, Jr., Vasan RS. Evaluating the added predictive ability of a new marker: from area under the ROC curve to reclassification and beyond. *Stat Med.* 2008;27(2):157-172; discussion 207-112.

39. Cook NR. Use and misuse of the receiver operating characteristic curve in risk prediction. *Circulation.* 2007;115(7):928-935.

40. Harrell FEJ. *Regression modeling strategies.* New York: Springer; 2001.

41. Libby P. Inflammation in atherosclerosis. *Arterioscler Thromb Vasc Biol.* 2012;32(9):2045-2051.

42. Baldus S, Heeschen C, Meinertz T, et al. Myeloperoxidase serum levels predict risk in patients with acute coronary syndromes. *Circulation.* 2003;108(12):1440-1445.

43. Buffon A, Biasucci LM, Liuzzo G, D'Onofrio G, Crea F, Maseri A. Widespread coronary inflammation in unstable angina. *N Engl J Med.* 2002;347(1):5-12.

44. Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. *Nature medicine.* 2011;17(11):1410-1422.

45. Della Bona R, Cardillo MT, Leo M, et al. Polymorphonuclear neutrophils and instability of the atherosclerotic plaque: a causative role? *Inflamm Res.* 2013;62(6):537-550.

46. Soehnlein O. Multiple roles for neutrophils in atherosclerosis. *Circ Res.* 2012;110(6):875-888.

47. Doring Y, Drechsler M, Wantha S, et al. Lack of neutrophil-derived CRAMP reduces atherosclerosis in mice. *Circ Res.* 2012;110(8):1052-1056.

48. Vogl T, Ludwig S, Goebeler M, et al. MRP8 and MRP14 control microtubule reorganization during transendothelial migration of phagocytes. *Blood.* 2004;104(13):4260-4268.

49. Hofmann MA, Drury S, Fu C, et al. RAGE mediates a novel proinflammatory axis: a central cell surface receptor for S100/calgranulin polypeptides. *Cell.* 1999;97(7):889-901.

50. Nagareddy PR, Murphy AJ, Stirzaker RA, et al. Hyperglycemia promotes myelopoiesis and impairs the resolution of atherosclerosis. *Cell Metab.* 2013;17(5):695-708.

51. Croce K, Gao H, Wang Y, et al. Myeloid-related protein-8/14 is critical for the biological response to vascular injury. *Circulation.* 2009;120(5):427-436.

52. Viemann D, Barczyk K, Vogl T, et al. MRP8/MRP14 impairs endothelial integrity and induces a caspase-dependent and -independent cell death program. *Blood.* 2007;109(6):2453-2460.

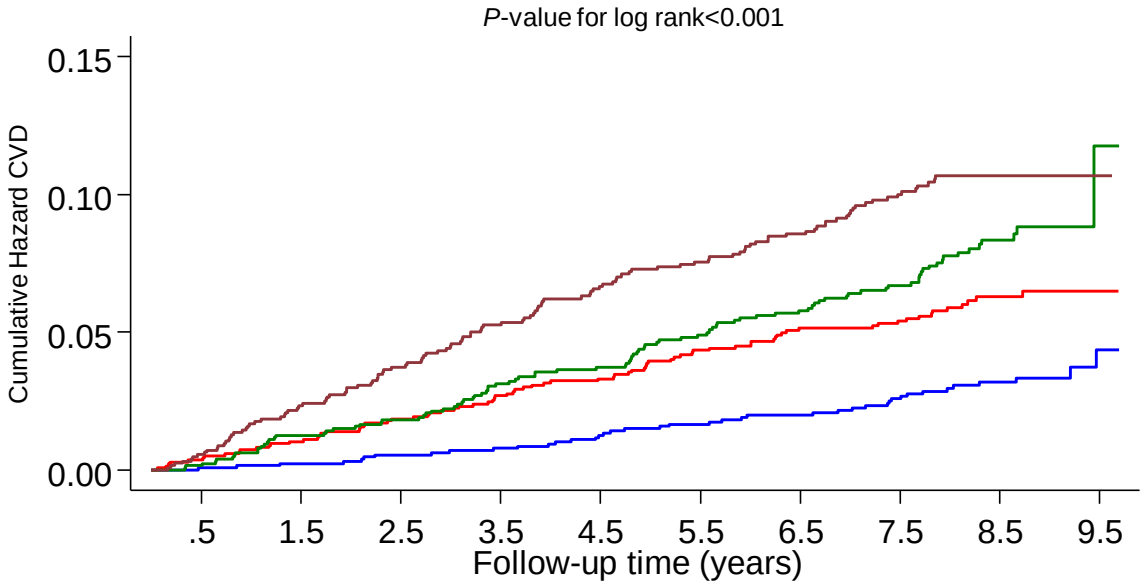
53. Viemann D, Strey A, Janning A, et al. Myeloid-related proteins 8 and 14 induce a specific inflammatory response in human microvascular endothelial cells. *Blood.* 2005;105(7):2955-2962.

54. Bjork P, Bjork A, Vogl T, et al. Identification of human S100A9 as a novel target for treatment of autoimmune disease via binding to quinoline-3-carboxamides. *PLoS Biol.* 2009;7(4):e97.

55. Bengtsson AA, Sturfelt G, Lood C, et al. Pharmacokinetics, tolerability, and preliminary efficacy of paquinimod (ABR-215757), a new quinoline-3-carboxamide derivative: studies in lupus-prone mice and a multicenter, randomized, double-blind, placebo-controlled, repeat-dose, dose-ranging study in patients with systemic lupus erythematosus. *Arthritis Rheum.* 2012;64(5):1579-1588.

Figure Legends

Figure 1. Cumulative Kaplan-Meier curves for cardiovascular disease during follow-up according to quartiles of calprotectin

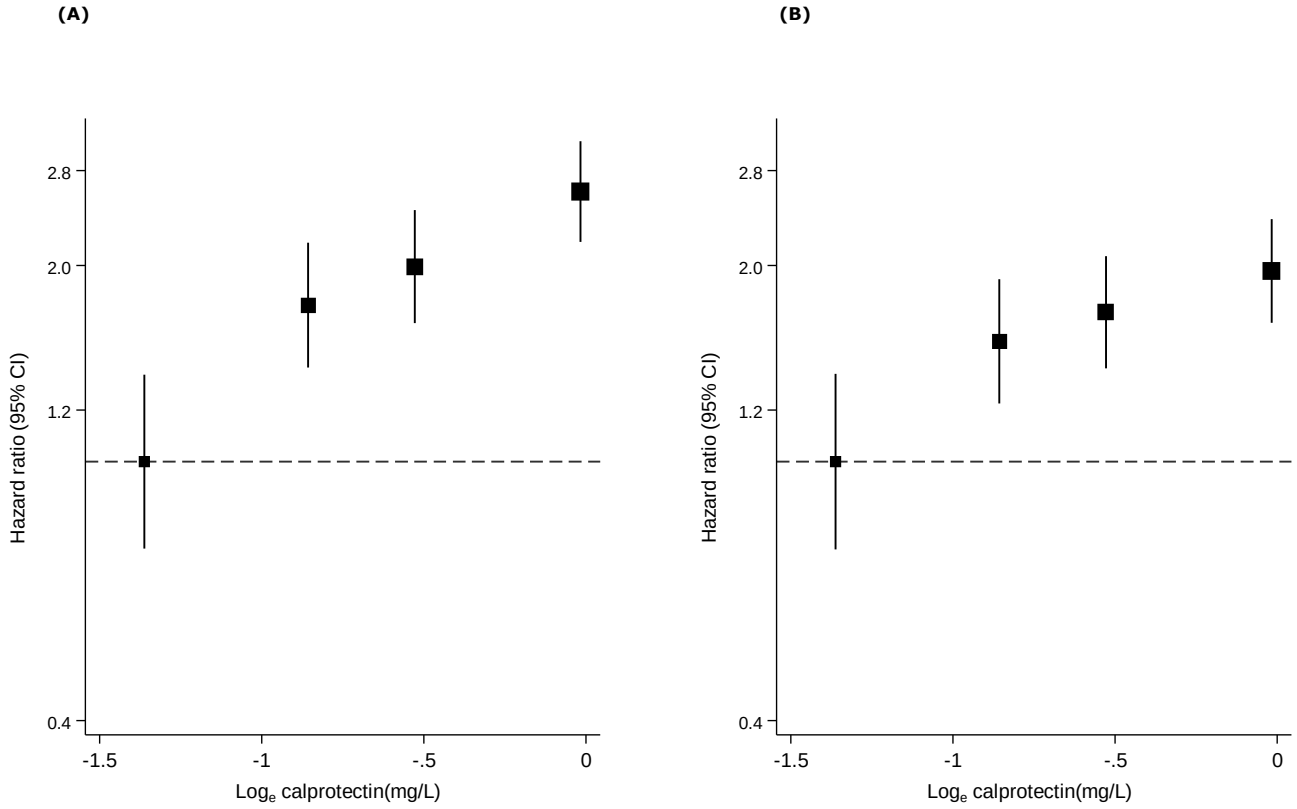


Number at risk

Quartile 1	1305	1297	1279	1267	1249	1236	1210	1149	727	146
Quartile 2	1368	1350	1322	1298	1281	1245	1218	1146	598	25
Quartile 3	1287	1261	1238	1208	1188	1146	1120	1048	515	13
Quartile 4	1256	1228	1194	1161	1128	1106	1067	978	436	10

CVD, cardiovascular disease

Figure 2. Hazard ratios for incident cardiovascular disease, by baseline concentrations of plasma calprotectin using floating absolute risks



A, Hazard ratios were adjusted for age and sex; **B**, adjustment in A plus smoking status, history of diabetes, systolic blood pressure, total cholesterol, and high-density lipoprotein-cholesterol

Table 1. Baseline participant characteristics overall and according to cardiovascular disease development

	Overall (N=5290) Mean (SD) or median (IQR) or n (%)	**Without CVD (N=4951) Mean (SD) median (IQR) or n (%)**	**With CVD (N=339) Mean (SD) or median (IQR) or n (%)**	***p*-value**
Plasma calprotectin (mg/L)	0.50 (0.35-0.70)	0.49 (0.35-0.69)	0.60 (0.44-0.85)	< 0.001
Questionnaire				
Male	2530 (47.8)	2288 (46.2)	242 (71.4)	< 0.001
Age at survey (years)	53 (12)	52 (12)	63 (11)	< 0.001
History of diabetes	282 (5.3)	240 (4.9)	42 (12.4)	< 0.001
Current smokers	1456 (27.5)	1345 (27.2)	111 (32.7)	< 0.001
Alcohol consumers	4001 (75.6)	3768 (76.1)	233 (68.7)	0.002
Use of anti-hypertensive medication	770 (15.5)	657 (14.2)	113 (34.4)	< 0.001
Use of lipid-lowering medication	126 (2.9)	105 (2.6)	21 (7.0)	< 0.001
Physical measurements				
BMI (kg/m^2)	26.5 (4.3)	26.4 (4.3)	27.7 (3.9)	< 0.001
SBP (mmHg)	126 (18)	125 (18)	140 (20)	< 0.001
DBP (mmHg)	73 (9)	73 (9)	79 (9)	< 0.001
Lipid markers				
Total cholesterol (mmol/l)	5.47 (1.04)	5.45 (1.04)	5.77 (1.10)	< 0.001
HDL-C (mmol/l)	1.27 (0.31)	1.28 (0.31)	1.16 (0.29)	< 0.001
Triglycerides (mmol/l)	1.11 (0.80-1.59)	1.09 (0.79-1.57)	1.33 (0.98-1.87)	< 0.001
Metabolic, inflammatory, and renal function markers				
hsCRP (mg/l)	1.30 (0.61-2.89)	1.25 (0.59-2.79)	2.22 (1.04-4.73)	< 0.001
Fasting plasma glucose (mmol/l)	5.00 (1.10)	4.97 (1.07)	5.38 (1.42)	<0.001
Creatinine (µmol/l)	71.0 (62.0-80.0)	70.5 (62.0-79.0)	76.0 (67.0-89.0)	< 0.001
Cystatin C (mg/dl)	0.90 (0.20)	0.89 (0.18)	1.04 (0.34)	< 0.001
eGFR (ml/min/1.73 m^2)	92.7 (16.7)	93.5 (16.2)	80.8 (19.1)	< 0.001

Continuous variables are reported as mean ± SD or median (interquartile range) and categorical variables are reported as n (%); BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; hsCRP, high sensitivity C-reactive protein; IQR, interquartile range; SBP, systolic blood pressure; SD, standard deviation.

Table 2. Cross-sectional Correlates of Plasma Calprotectin

	Partial correlation r (95% CI)[a]	Percentage difference (95% CI) in calprotectin levels per 1 SD higher or compared to reference category of correlate[b]
Log_e calprotectin (mg/L)	-	-
Sex		
Female	-	Ref
Male	-	9% (6, 13)**
Questionnaire		
Age at survey (years)	0.11 (0.09, 0.14)***	6% (5, 8)***
History of diabetes		
No	-	Ref
Yes	-	9% (2, 17)
Smoking status		
Never and former smokers	-	Ref
Current smokers	-	22% (18, 26)***
Alcohol consumption		
Non-consumers	-	Ref
Current consumers	-	-7% (-10, -4)***
Use of anti-hypertensive medication		
No	-	Ref
Yes	-	11% (6, 16)***
Use of lipid-lowering medication		
No		Ref
Yes		9% (-1, 19)
Physical measurements		
BMI (kg/m^2)	0.17 (0.14, 0.19)***	9% (8, 11)***
SBP (mmHg)	0.14 (0.11, 0.16)***	9% (7, 11)***
DBP (mmHg)	0.10 (0.08, 0.13)***	6% (5, 8)***
Lipid markers		
Total cholesterol (mmol/l)	0.08 (0.06, 0.11)***	5% (3, 6)***
HDL-C (mmol/l)	-0.15 (-0.18, -0.13)***	-9% (-10, -7)***
Triglycerides (mmol/l)	0.10 (0.07, 0.13)***	6% (4, 7)***
Metabolic, inflammatory, and renal function markers		
hsCRP (mg/l)	0.42 (0.40, 0.44)***	26% (24, 27)***
Fasting plasma glucose (mmol/l)	0.05 (0.02, 0.08)***	3% (1, 4)**
Creatinine (µmol/l)	0.02 (-0.01, 0.05)	1% (-0, 3)
Cystatin C (mg/dl)	0.13 (0.11, 0.16)***	8% (7, 10)***
eGFR ($ml/min/1.73\ m^2$)	-0.11 (-0.14, -0.09)***	-8% (-9, -6)***

BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation); HDL-C, high-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; Ref, reference; SD, standard deviation; SBP, systolic blood pressure;

Asterisks indicate the level of statistical significance: *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; [a], Partial correlation coefficients log_e between calprotectin and the row variables; [b], Percentage change in log_e calprotectin levels per 1 SD increase in the row variable (or for categorical variables, the percentage difference in mean log_e calprotectin levels for the category versus the reference) adjusted for age and sex.

Table 3. Prospective Associations of Plasma Calprotectin With Risk of Cardiovascular Disease

Calprotectin	Events/ Total	Model 1		Model 2		Model 3		Model 4	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
Cardiovascular disease									
Per 1 SD increase	339 / 5290	1.38 (1.25 to 1.54)	< 0.001	1.26 (1.13 to 1.41)	< 0.001	1.24 (1.11 to 1.39)	< 0.001	1.15 (1.02 to 1.30)	0.027
Quartile 1 (0.02-0.35)	41 / 1333	ref		ref		ref		ref	
Quartile 2 (0.36-0.50)	79 / 1382	1.74 (1.19 to 2.54)	0.004	1.53 (1.05 to 2.24)	0.028	1.51 (1.03 to 2.20)	0.034	1.41 (0.96 to 2.07)	0.076
Quartile 3 (0.51-0.70)	97 / 1308	1.99 (1.38 to 2.88)	< 0.001	1.70 (1.17 to 2.45)	0.005	1.67 (1.15 to 2.41)	0.007	1.52 (1.05 to 2.21)	0.028
Quartile 4 (0.71-13.23)	122 / 1277	2.60 (1.82 to 3.71)	< 0.001	1.96 (1.37 to 2.82)	< 0.001	1.89 (1.31 to 2.72)	0.001	1.56 (1.07 to 2.29)	0.022
***p*-value for trend**			< 0.001		< 0.001		< 0.001		0.037
Coronary heart disease									
Per 1 SD increase	246 / 5290	1.38 (1.22 to 1.56)	< 0.001	1.23 (1.08 to 1.41)	0.002	1.21 (1.06 to 1.38)	0.005	1.19 (1.03 to 1.38)	0.020
Quartile 1 (0.02-0.35)	29 / 1323	ref		ref		ref		ref	
Quartile 2 (0.36-0.50)	53 / 1382	1.65 (1.05 to 2.60)	0.031	1.41 (0.90 to 2.23)	0.136	1.40 (0.88 to 2.21)	0.151	1.37 (0.87 to 2.17)	0.177
Quartile 3 (0.51-0.70)	77 / 1308	2.28 (1.48 to 3.51)	< 0.001	1.89 (1.22 to 2.91)	0.004	1.84 (1.19 to 2.85)	0.006	1.79 (1.16 to 2.78)	0.009
Quartile 4 (0.71-13.23)	87 / 1277	2.62 (1.72 to 4.01)	< 0.001	1.91 (1.24 to 2.94)	0.003	1.83 (1.18 to 2.82)	0.007	1.73 (1.09 to 2.73)	0.019
***p*-value for trend**			< 0.001		0.002		0.004		0.013
Stroke									
Per 1 SD increase	91 / 5290	1.25 (1.02 to 1.54)	0.035	1.19 (0.96 to 1.48)	0.117	1.17 (0.94 to 1.45)	0.173	0.96 (0.75 to 1.21)	0.710
Quartile 1 (0.02-0.35)	13 / 1323	ref		ref		ref		ref	
Quartile 2 (0.36-0.50)	26 / 1382	1.81 (0.93 to 3.53)	0.082	1.69 (0.86 to 3.31)	0.125	1.66 (0.85 to 3.25)	0.140	1.39 (0.71 to 2.74)	0.338
Quartile 3 (0.51-0.70)	22 / 1308	1.43 (0.72 to 2.84)	0.313	1.31 (0.65 to 2.62)	0.452	1.28 (0.64 to 2.56)	0.491	1.01 (0.50 to 2.05)	0.969
Quartile 4 (0.71-13.23)	30 /1277	1.94 (1.01 to 3.75)	0.047	1.67 (0.86 to 3.27)	0.132	1.59 (0.81 to 3.12)	0.180	0.99 (0.48 to 2.02)	0.970
***p*-value for trend**			0.111		0.288		0.375		0.552
Ischemic stroke									
Per 1 SD increase	70 / 5290	1.27 (1.00 to 1.61)	0.047	1.19 (0.93 to 1.53)	0.162	1.15 (0.90 to 1.49)	0.265	0.92 (0.70 to 1.21)	0.532
Quartile 1 (0.02-0.35)	10 / 1323	ref		ref		ref		ref	
Quartile 2 (0.36-0.50)	19 / 1382	1.66 (0.77 to 3.58)	0.193	1.52 (0.71 to 3.30)	0.283	1.50 (0.69 to 3.24)	0.306	1.21 (0.55 to 2.64)	0.633
Quartile 3 (0.51-0.70)	17 / 1308	1.36 (0.62 to 2.97)	0.447	1.21 (0.55 to 2.66)	0.634	1.17 (0.53 to 2.59)	0.691	0.89 (0.40 to 2.00)	0.786
Quartile 4 (0.71-13.23)	24 / 1277	1.89 (0.90 to 3.96)	0.092	1.56 (0.73 to 3.32)	0.246	1.44 (0.67 to 3.09)	0.349	0.82 (0.36 to 1.85)	0.636
***p*-value for trend**			0.158		0.397		0.551		0.367

CI, confidence interval; HR, hazard ratio
Model 1: Age and sex
Model 2: Model 1 plus smoking status, history of diabetes, systolic blood pressure, total cholesterol, and high-density lipoprotein-cholesterol
Model 3: Model 2 plus triglycerides, body mass index, alcohol consumption, glucose, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation)
Model 4: Model 3 plus $\log_e$ high sensitivity C-reactive protein

Appendix 1. STROBE 2007 Statement—Checklist of items that should be included in reports of cohort studies

Section/Topic	Item #	Recommendation	Reported on page #
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Page 1
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Page 2
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Page 4
Objectives	3	State specific objectives, including any prespecified hypotheses	Page 4
Methods			
Study design	4	Present key elements of study design early in the paper	Study design and population
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Study design and population
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	Study design and population
		(*b*) For matched studies, give matching criteria and number of exposed and unexposed	Not applicable
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Assessment of calprotectin and risk markers
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	Assessment of calprotectin and risk markers

Bias	9	Describe any efforts to address potential sources of bias	Statistical analyses
Study size	10	Explain how the study size was arrived at	Statistical analyses
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	Statistical analyses
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Statistical analyses
		(*b*) Describe any methods used to examine subgroups and interactions	Statistical analyses
		(*c*) Explain how missing data were addressed	Not applicable
		(*d*) If applicable, explain how loss to follow-up was addressed	Not applicable
		(*e*) Describe any sensitivity analyses	Statistical analyses
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Study design and population
		(b) Give reasons for non-participation at each stage	Study design and population
		(c) Consider use of a flow diagram	Study design and population
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Results; Tables 1-2
		(b) Indicate number of participants with missing data for each variable of interest	
		(c) Summarise follow-up time (eg, average and total amount)	Results

Outcome data	15*	Report numbers of outcome events or summary measures over time	Results
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results; Table 3
		(*b*) Report category boundaries when continuous variables were categorized	Results; Table 3
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	Results; Appendices 2-4
Discussion			
Key results	18	Summarise key results with reference to study objectives	Discussion
Limitations			
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Discussion
Generalisability	21	Discuss the generalisability (external validity) of the study results	Discussion
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	Pages 15-16

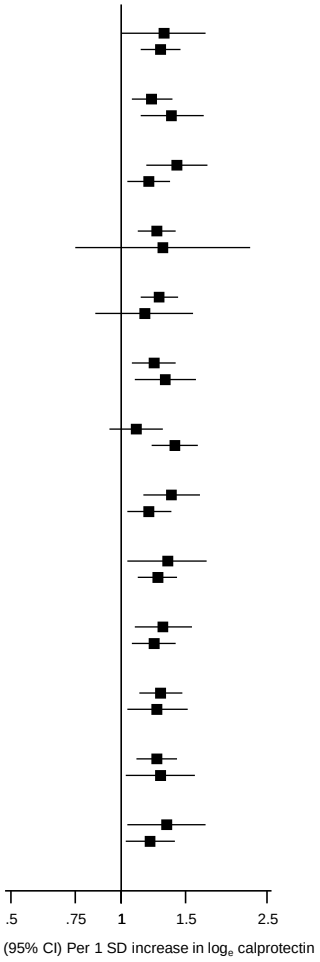
Appendix 2. Prospective Associations of Plasma Calprotectin With Risk of Cardiovascular Disease, on Exclusion of First Two Years of Follow-up, People With History of Diabetes Mellitus, and Use of Lipid-lowering Medication

Exclusions	Events/ Total	Model 1		Model 2		Model 3		Model 4	
		HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value	HR (95% CI)	*p*-value
				Cardiovascular disease					
First two years of follow-up	260 / 5097	1.31 (1.16 to 1.48)	< 0.001	1.20 (1.05 to 1.36)	0.006	1.18 (1.04 to 1.35)	0.012	1.11 (0.96 to 1.28)	0.161
People with a history of diabetes	297 / 5008	1.41 (1.26 to 1.58)	< 0.001	1.27 (1.13 to 1.53)	< 0.001	1.25 (1.11 to 1.42)	< 0.001	1.16 (1.02 to 1.33)	0.026
Use of lipid-lowering medication	318 / 5164	1.38 (1.24 to 1.54)	< 0.001	1.25 (1.12 to 1.41)	0.001	1.24 (1.10 to 1.39)	< 0.001	1.14 (1.00 to 1.29)	0.051

CI, confidence interval; HR, hazard ratio; hazard ratios are reported per 1 standard deviation (SD) increase in $\log_e$ calprotectin
Model 1: Age and sex
Model 2: Model 1 plus smoking status, history of diabetes, systolic blood pressure, total cholesterol, and high-density lipoprotein-cholesterol
Model 3: Model 2 plus triglycerides, body mass index, alcohol consumption, glucose, and estimated glomerular filtration rate (as calculated using the Chronic Kidney Disease Epidemiology Collaboration combined creatinine-cystatin C equation)
Model 4: Model 3 plus $\log_e$ high sensitivity C-reactive protein

Appendix 3. Hazard ratios for calprotectin and cardiovascular disease risk by several participant level characteristics

Subgroup	No. of participants	No. of CVD events	HR (95% CI)	*P*-value*
Age at survey (years)				
< 50	2,362	47	1.31 (1.00, 1.70)	.888
≥ 50	2,928	292	1.28 (1.13, 1.45)	
Sex				
Males	2,530	242	1.21 (1.07, 1.38)	.307
Females	2,760	97	1.37 (1.13, 1.68)	
Alcohol consumption				
Non-alcohol consumers	1289	106	1.42 (1.17, 1.72)	.139
Alcohol consumers	4,001	233	1.19 (1.04, 1.36)	
Use of statins				
No	4,211	280	1.25 (1.11, 1.41)	.896
Yes	126	21	1.30 (0.75, 2.25)	
History of diabetes				
No	5,008	297	1.27 (1.13, 1.43)	.558
Yes	282	42	1.16 (0.85, 1.57)	
Smoking status				
No	3,834	228	1.23 (1.07, 1.41)	.542
Yes	1,456	111	1.32 (1.09, 1.60)	
Fasting glucose (mmol/l)				
< 5.0	3,006	147	1.10 (0.93, 1.30)	.032
≥ 5.0	2,284	192	1.40 (1.21, 1.62)	
Body mass index (kg/m2)				
< 25.99	2,647	120	1.37 (1.15, 1.64)	.228
≥ 25.99	2,643	219	1.19 (1.04, 1.37)	
Systolic blood pressure (mmHg)				
< 124	2,736	67	1.34 (1.04, 1.71)	.665
≥ 124	2,554	272	1.26 (1.11, 1.42)	
Total cholesterol (mmol/l)				
< 5.41	2,653	126	1.30 (1.09, 1.56)	.612
≥ 5.41	2,637	213	1.23 (1.07, 1.41)	
HDL-cholesterol (mmol/l)				
< 1.24	2,653	220	1.28 (1.12, 1.47)	.851
≥ 1.24	2,637	119	1.25 (1.04, 1.52)	
Estimated GFR (ml/min/1.73 m2)				
< 94.35	2,645	250	1.25 (1.10, 1.42)	.853
≥ 94.35	2,645	89	1.28 (1.03, 1.59)	
hsC-reactive protein (mg/l)				
< 1.30	2,334	88	1.33 (1.04, 1.70)	.476
≥ 1.30	2,334	194	1.20 (1.03, 1.40)	



Hazard ratios were adjusted for age, sex, smoking status, history of diabetes, systolic blood pressure, total cholesterol, and high-density lipoprotein-cholesterol; CI, confidence interval (bars); CVD, cardiovascular disease; GFR, glomerular filtration rate; HDL, high-density lipoprotein; HR, hazard ratio; hs, high sensitivity; SD, standard deviation; *, *p*-value for interaction; cut-offs used for fasting glucose, body mass index, systolic blood pressure, total cholesterol, HDL-cholesterol, estimated GFR, and hsC-reactive protein are median values.

Appendix 4. Direct Comparisons Within the PREVEND Study of Associations of Several
Established and Emerging Cardiovascular Risk Markers With Risk of Cardiovascular Disease

Risk marker	Hazard Ratio (95% CI)	*p*-value
Mean age at time of survey (years)	2.15 (1.89 to 2.45)	< 0.001
Current smoker	1.79 (1.32 to 2.42)	< 0.001
Systolic blood pressure (mmHg)	1.37 (1.25 to 1.51)	< 0.001
History of diabetes	1.35 (0.97 to 1.88)	0.078
Total cholesterol (mmol/l)	1.26 (1.13 to 1.40)	< 0.001
High-density lipoprotein cholesterol (mmol/l)	0.79 (0.70 to 0.90)	< 0.001
Triglycerides (mmol/l)	1.03 (0.94 to 1.12)	0.540
High sensitivity C-reactive protein (mg/l)	1.30 (1.15 to 1.47)	< 0.001

Analyses are based on 5290 participants with 339 first-ever cardiovascular disease events. Hazard ratios were calculated per 1 standard deviation increment in measured level or as compared with the relevant reference category.
Where appropriate, hazard ratios were adjusted for age, sex, plus smoking status, history of diabetes, systolic blood pressure,
total cholesterol, and high-density lipoprotein-cholesterol